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Polyamine Regulation of Plasma Membrane Phospholipid Flip-Flop during Apoptosis*

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During apoptosis, phosphatidylserine (PS) is moved from the plasma membrane inner leaflet to the outer leaflet where it triggers recognition and phagocytosis of the apoptotic cell. Although the mechanisms of PS appearance during apoptosis are not well understood, it is thought that declining activity of the aminophospholipid translocase and calcium-mediated, nonspecific flip-flop of phospholipids play a role. As previous studies in the erythrocyte ghost have shown that polyamines can alter flip-flop of phospholipids, we asked whether alterations in cellular polyamines in intact cells undergoing apoptosis would affect PS appearance, either by altering aminophospholipid translocase activity or phospholipid flip-flop. Cells of the human leukemic cell line, HL-60, were incubated with or without the ornithine decarboxylase inhibitor, difluoromethylornithine (DFMO), and induced to undergo apoptosis by ultraviolet irradiation. Whereas DFMO treatment resulted in profound depletion of putrescine and spermidine (but not spermine), it had no effect on caspase activity, DNA fragmentation, or plasma membrane vesiculation, typical characteristics of apoptosis. Notably, DFMO treatment prior to ultraviolet irradiation did not alter the decline in PS inward movement by the aminophospholipid translocase as measured by the uptake of 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl] (NBD)-labeled PS detected in the flow cytometer. Conversely, the appearance of endogenous PS in the plasma membrane outer leaflet detected with fluorescein isothiocyanate-labeled annexin V and enhanced phospholipid flip-flop detected by the uptake of 1-palmitoyl-1-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)aminocaproyl]-*sn*-glycero-3-phosphocholine (NBD-PC) seen during apoptosis were significantly inhibited by prior DFMO treatment. Importantly, replenishment of spermidine, by treatment with exogenous putrescine to bypass the metabolic blockade by DFMO, restored both enhanced phospholipid flip-flop and appearance of PS during apoptosis. Such restoration was seen even in the presence of cycloheximide but was not seen when polyamines were added externally just prior to assay. Taken together, these data show that intracellular polyamines can modulate PS appearance resulting from nonspecific flip-flop of phospholipids across the plasma membrane during apoptosis.

Under normal conditions, plasma membrane phospholipids of cells appear to be asymmetrically distributed across the bilayer with phosphatidylserine found almost entirely in the inner leaflet and sphingomyelin in the outer leaflet of the bilayer (1–3). Maintenance of this asymmetry is attributed largely to the activity of the aminophospholipid translocase that moves phosphatidylserine (PS)[1] and, with a lesser affinity, phosphatidylethanolamine from the outer to the inner leaflet (4–7). On the other hand, the appearance of PS, seen as a universal feature of apoptosis (3, 8, 9), and phospholipid flip-flop during inflammatory cell activation (10–13) require enhanced, nonspecific (with regard to head group) transbilayer movement of phospholipids. This nonspecific transbilayer movement, or flip-flop, of phospholipids appears to be calcium-dependent as demonstrated in cells (neutrophils, HL-60s, Jurkats, and U937 promonocytes) undergoing apoptosis (8, 14), ionophore-treated erythrocytes/erythrocyte ghosts (15, 16), and activated platelets (10, 11). Although not yet defined, targets potentially relevant to enhanced plasma membrane flip-flop include several integral membrane proteins, "flippases" or "scramblases" (17–20), but also intracellular proteases (calpain and the caspases) (9, 21–24) and transglutaminase (12, 25, 26) that may alter membrane tethering by the submembranous cytoskeleton. Additionally, the anionic phospholipid phosphatidylinositol bisphosphate may play a role (27, 28). Of note, in both the calcium-treated erythrocyte ghost (15) and the phosphatidylinositol bisphosphate-loaded erythrocyte membrane or vesicle (28), exogenously added polyamines, particularly spermine, have been shown to antagonize calcium-induced nonspecific phospholipid flip-flop. These observations in simplified models of phospholipid flip-flop, prompted us to ask whether polyamines in intact cells might govern the phospholipid flip-flop seen during apoptosis. By using the ornithine decarboxylase inhibitor, DFMO, to disrupt polyamine biosynthesis, we tested each of the processes involved in transbilayer movement of phospholipids during cellular apoptosis. As the data show, DFMO treatment of HL-60s resulted in characteristic progressive depletion of intracellular spermidine and putrescine, with sparing of spermine levels. Whereas polyamine depletion with DFMO did not alter the decline in aminophospholipid translocase activity, inhibition of PS appearance and phospholipid flip-flop was demonstrated with alterations in intracellular polyamines during apoptosis. Significantly, alteration in polyamine levels in DFMO-treated cells inhibited only the events previously shown to require extracellular calcium

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[1] The abbreviations used are: PS, phosphatidylserine; DFMO, 2-(difluoromethyl)-DL-ornithine monohydrochloride monohydrate; FITC, fluorescein isothiocyanate; NBD-PC, 1-palmitoyl-1-[6-[(7-nitro-2–1,3-benzoxadiazol-4-yl]aminocaproyl]-*sn*-glycero-3-phosphocholine; HBS, HEPES-buffered saline; BSA, bovine serum albumin; PCR, polymerase chain reaction; CHAPS, 3-[(3-cholamidopropyl)dimethylammoniol]-1-propanesulfonic; acid; PBS, phosphate-buffered saline; AFC, 7-amino-4-trifluoromethylcoumarin.

(8). DFMO treatment had no effect on calcium mobilization, scramblase or Bcl-2 expression, caspase or transglutaminase activity, plasma membrane vesiculation, or DNA fragmentation. Importantly, repletion of spermidine levels by the addition of putrescine to cell cultures restored phospholipid flip-flop and the appearance of PS in the plasma membrane outer leaflet during apoptosis. This restoration required intracellular repletion of spermidine, as external addition of the various polyamines did not restore PS appearance or phospholipid flip-flop. These findings demonstrate that phospholipid flip-flop with resulting PS appearance is dissociable from other events of apoptosis and can be governed by cellular polyamines.

EXPERIMENTAL PROCEDURES

Cell Culture

The human leukemia cell line HL-60 was obtained from ATCC (Rockville, MD) and cultured in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 20% heat-inactivated fetal bovine serum (Gemini Biological Products, Inc., Calabasas, CA) and maintained in the undifferentiated state at 37 °C in a 5% CO_2, humidified atmosphere. For polyamine depletion experiments, HL-60 cells were plated at a cell density of 1.25×10^5 cells/ml in RPMI with 20% fetal calf serum and treated with 1 mM DFMO ((2-(difluoromethyl)-DL-ornithine monohydrochloride monohydrate), a generous gift of Dr. Ekkehard Böhme, Hoechst-Roussel, Cincinnati, OH) and incubated 120 h at 37 °C in a 5% CO_2 humidified atmosphere. Reconstitution of polyamines was accomplished by the addition of putrescine in RPMI at a final concentration of 10 μM at 72 and 96 h post-seeding. Control cells were seeded at 6.25×10^4 cells/ml to accommodate a faster growth rate and were incubated under identical conditions at the same time as the DFMO-treated cells. All cultures were harvested while at approximately $1–1.5 \times 10^6$ cells/ml. In experiments in which protein synthesis was inhibited by cycloheximide, the inhibitor was added at a final concentration of 0.5 μg/ml concurrently with the initial addition of putrescine. The cells were then incubated approximately 20 h before harvesting. This concentration and incubation period was shown to be sufficient to stop most of protein synthesis as determined by a 75% reduction in [^{35}S]Cys incorporation but which resulted in less than 10% cell death as determined by trypan blue staining.

Experimental Conditions

Cells were pelleted, resuspended in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 0.25% BSA, with or without 5 μM cytochalasin D (see below) at 2×10^6 cells/ml, and plated in 6- or 12-well plates. Apoptosis was induced by UV irradiation at 254 nm for 5 min, and the cells were then incubated at 37 °C in a 5% CO_2 humidified atmosphere for 2 h to allow the apoptotic phenotypes to develop (8). At the end of the incubation period, cell cultures were subdivided, and samples were simultaneously stained for surface phosphatidylserine (PS) and phospholipid uptake and prepared for polyamine analysis and DNA fragmentation (see below). Where noted, polyamines were added externally to assess the effect on PS appearance and phospholipid flip-flop. In these experiments, either spermidine, putrescine, or spermine was added to a final concentration of 10 μM, 5 min before the uptake procedure commenced. The cells were maintained in the presence of the exogenous polyamines throughout the uptake procedure. Alternatively, cells were prepared as above for the determination of scramblase mRNA, scramblase or Bcl-2 Western blot, or caspase or transglutaminase activity as described below.

Flow Cytometry

Propidium Iodide Staining for Apoptotic DNA—DNA degradation was determined by the appearance of a hypodiploid fraction in permeabilized propidium iodide-stained cells as described previously (8). Nuclear changes were confirmed microscopically, and DNA fragmentation was identified by characteristic "laddering" in agarose gels (29).

PS Detection—Cells expressing PS in the plasma membrane outer leaflet were identified as those binding FITC-labeled annexin V using an Apoptosis Detection Kit (R & D Systems, Minneapolis, MN) using the manufacturer's recommendations. Under control conditions, the binding of FITC-labeled annexin V to phosphatidylserine on the surface of apoptotic HL-60 cells closely correlates with the appearance of nuclear and cytoplasmic condensation by light microscopy and the appearance of hypodiploid DNA as described previously (8). The cells were analyzed on a Coulter XL (Miami, FL) flow cytometer, and the results were analyzed with PC-LYSYS software (Becton Dickinson, Franklin Lakes, NJ). Annexin-positive cells were determined as described in the Apoptosis Kit by setting quadrants to separate viable cells from PI-permeant cells and non-apoptotic cells from those staining highly for the FITC-labeled annexin V probe. Percent of cells positive for PS appearance was determined from the cells staining greater than the control population threshold. Mean fluorescence of the PI-impermeant cells was simultaneously determined.

Phospholipid Uptake—Phospholipid uptake was carried out in HEPES-buffered saline (HBS) (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4, with 1 mM $CaCl_2$). Following the incubation period, cells were harvested, washed once, and resuspended in HBS (1×10^7 cells/ml). NBD-labeled phospholipids (Avanti Polar Lipids, Alabaster, AL) were prepared by drying 1 μg of 1-palmitoyl-1-(6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)aminocaproyl]-*sn*-glycero-3-phosphocholine (NBD-PC) or 1 μg 1-palmitoyl-1-(6-[(7-nitro-2–1, 3-benzoxadiazol-4-yl)aminocaproyl]-*sn*-glycero-3-phosphoserine (NBD-PS) in a glass tube. The lipids were resuspended in 0.25% BSA with 200 μM phenylmethylsulfonyl fluoride in HBS to a final concentration of 50 μg/ml. Previous studies have shown that these NBD-labeled probe lipids are readily solubilized in aqueous media containing 0.25% albumin and will partition into the plasma membrane outer leaflet (7, 12, 15). The cells (5×10^5 in 50 μl) were incubated with 1 μl of the lipid suspension and 5 μl of 50 mg/ml propidium iodide for 10 min at room temperature. Albumin extraction of the plasma membrane outer leaflet to remove probe lipid that had not entered the cell was performed (7, 12, 15) with 50 μl of 1% BSA in HBS for an additional 5 min. The samples were diluted with 900 μl of ice-cold HBS, transferred to an ice bath, and analyzed in the cytofluorograph within 2 h. Early experiments demonstrated that the signal from the cell-associated lipid is stable during this time frame. The mean fluorescence values of the phospholipid uptakes were determined by setting quadrants in such a manner as to separate cells staining positively for propidium iodide (dead or highly permeant cells) from viable cell populations. As in previous studies (4, 6), NBD-PS was taken up quickly and was largely unavailable for albumin extraction in control cells, as is characteristic of cells demonstrating aminophospholipid translocase activity. In contrast to the uptake of NBD-PS, the uptake of NBD-PC, a lipid probe not transported by the aminophospholipid translocase was used as a marker for "nonspecific" transbilayer movement of phospholipids that we and others (8, 10, 11, 14–16) have shown to be dependent on calcium minimally by control cells and remained largely available for albumin extraction (4, 6, 7, 10–12, 15, 30). During apoptosis, uptake of NBD-PS declines and NBD-PC increases as has been described previously (7, 8). Metabolism of the NBD-labeled probes did not occur during the incubation as demonstrated by TLC of cellular lipids extracted by the acidified Bligh and Dyer method used previously (13).

In initial experiments, cells were incubated with NBD-labeled sphingomyelin (Molecular Probes, Eugene, OR) to measure endocytosis during apoptosis, as it is thought that this lipid is internalized by the endocytic route rather than by transbilayer flip-flop (31). For all conditions of incubation, we saw no evidence for enhanced uptake of NBD-SM. Additionally, endocytosis is inhibited by cytochalasin D. This agent was used to rule out further a role for endocytosis in the uptake of the probe lipids. The presence of cytochalasin D (5 μg/ml) (Sigma) during incubation did not alter the uptake of either NBD-PC or NBD-PS and so was omitted except where noted. Thus, having ruled out a role for endocytosis of the lipid probes, NBD-labeled PS and PC uptake was used as a measure of transbilayer phospholipid movement, either via aminophospholipid translocase (NBD-PS) or nonspecific flip-flop (NBD-PC), respectively (7, 12, 15).

Quantification of Intracellular Free Polyamines

At the end of the incubation period the HL-60s (1×10^6 cells in 0.5 ml) were separated from media by centrifugation, washed once in PBS, and resuspended in 300 μl of perchloric acid (5%). After incubation of the samples on ice for 1 h, samples were either derivatized immediately or frozen (−20 °C) until needed. Polyamines were quantified by reverse phase-high pressure liquid chromatography following dansyl chloride derivatization and precolumn clean up as previously published (13).

Phospholipid Analysis

Phospholipid analysis was accomplished by lipid extraction of 10^7 cells utilizing an acidified Bligh and Dyer extraction (13). The extracts

were then dried under nitrogen and resuspended in isopropyl alcohol, hexane, 0.2% ammonium acetate, pH 7.0, at a ratio of 58:40:2 (solvent A). The lipid classes were separated on a 5-μm silica analytical column (Lichrosorb; Phenomenex, Torrance, CA) utilizing a gradient running from 37.5 to 100% solvent B (isopropyl alcohol, hexane, 0.2% ammonium acetate, pH 7.0, at a ratio of 50:40 10) over a 20-min period at 1 ml/min. The lipid peaks were then collected and pooled representing the phosphatidylethanolamine-phosphatidylinositol, the phosphatidylserine, and the phosphatidylcholine peaks. Phosphorus analysis was then done on the pooled fractions utilizing the phosphorus assay of Gerlach and Deuticke. (32).

Calcium Mobilization

The effect of DFMO on the ability of cells to mobilize calcium was done by the method of Lennon *et al.* (33) using fura-2-acetoxymethyl ester.

Scramblase mRNA by Quantitative PCR

To determine the relative amounts of scramblase transcript in the cells after the various treatments, mRNA was quantified using Advantage RT-for-PCR Kit and PCR-MIMIC kit (both from CLONTECH Laboratories Inc., Palo Alto, CA). Briefly, cells were lysed and mRNA isolated using Trizol total RNA isolation reagent (Life Technologies, Inc.). mRNA was reverse-transcribed to cDNA using the Advantage RT-for-PCR kit. Determination of scramblase mRNA quantity was done using competitive PCR of the cDNA against a mimic DNA fragment constructed with sequences complementary to the scramblase-specific primers. These primers were 5′-GACAGCATTCCAAGGACCTCCAG-GATA-3′ and 5′-GCCTCTCTCAAAATTCCAGTCCAGTGC-3′ which would be expected to produce a DNA fragment corresponding to bases 59–787 of the scramblase sequence, a fragment 729 bases long. Mimic DNA fragment primers were 5′-GACAGCATTCCAAGGACCTCCAG-GATACGCAAGTGAAATCTCCTCCG-3′ and 5′-GCCTCTCTCAAAAT-TCCAGTCCAGTGCATTTGATTCTGGACCATGGC-3′ resulting in a fragment of 560 bases. The mimic DNA fragment was quantified and diluted to known concentrations. PCR was set up initially with the mimic DNA fragment at 1:10 dilutions and constant amounts of the cDNA. After PCR, the samples were run out on a 1% agarose gel, and concentration of the mimic DNA fragment which resulted in bands of approximately equal intensity was determined. To determine more accurately scramblase mRNA concentration, a second dilution series of the mimic DNA fragment was made from the next highest dilution relative to the one that produced bands of equal intensity. This 1:2 dilution series was then also amplified, and again the mimic DNA fragment concentration giving equivalent band intensity was determined. This dilution corresponded to an amount of mimic DNA fragment equal to the amount of scramblase cDNA and therefore the amount of scramblase mRNA in the initial preparation.

Caspase Activity Assay

Intracellular caspase activity was determined utilizing the caspase substrate *N*-carbobenzyloxy-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethylcoumarin (Enzyme Systems Products, Livermore, CA) which is a general substrate used to measure the activity of caspases 3, 6, 7, 8, and 10. Cells (10^6/assay) were washed 1× in PBS and resuspended to 1×10^8 cells/ml in 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol with 1 mM phenylmethylsulfonyl fluoride and frozen. The assay was done by mixing 2 ml of the assay buffer (100 mM HEPES, pH 7.2, 2 mM dithiothreitol, 0.1% CHAPS, and 1% sucrose) along with the AFC-peptide substrate at 100 mM in a cuvette and placed in an SLM 8000C spectrofluorometer with an excitation of 400 nm and an emission of 505 nm. Following the establishment of a baseline, the cell lysate (10 μl) was added, and the reaction was allowed to continue for 8 min. Caspase activity was determined as the emission value of the appearance of the free AFC group as it was cleaved from the peptide.

Transglutaminase Assay

The determination of transglutaminase activity was done as described previously (12).

Western Blotting

HL-60 cells (10^6) were harvested, pelleted, washed 1× in PBS, and resuspended in H_2O with 1 mg each of aprotinin, phenylmethylsulfonyl fluoride, and leupeptin (Sigma) and 1% Triton X-100. Following a 15-min incubation on ice, 5 μl of each sample was taken for total protein determination by the Bradford method (Bio-Rad). Alternatively, the cells were harvested, washed 1× in PBS, and lysed using nitrogen cavitation. Large cell debris was removed by centrifugation, and then membranes were pelleted by ultracentrifugation, and protein concentrations were determined using BCL protein assay kit (Pierce). For the blots, equivalent amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After overnight blocking in 1% BSA, the blots were probed with either anti-Bcl-2 (Calbiochem) at 1 mg/ml for 1 h at room temperature or with rabbit antibodies produced to a peptide (CESTGSQEQKSGVW) corresponding to the C-terminal extracellular portion of the scramblase gene for 2 h at room temperature (19). The blots were washed 3× in wash buffer (25 mM Tris, pH 7.8, 190 mM NaCl, and 0.2% Tween 20) and then probed with a horseradish peroxidase-conjugated secondary antibody. The labeled bands were detected using an ECL chemiluminescence Western blotting kit (Amersham Pharmacia Biotech).

Cell Volume Determination

Cell volumes were analyzed with a Coulter Counter ZM connected to a Channelyzer 256 (Coulter, Hialeah, FL).

Statistical Analysis

Data were analyzed using either a univariate or mixed effects repeated measures analysis of variance model. When analysis of variance indicated significance, the Tukey-Kramer HSD test for all pairs was used to compare groups.

RESULTS

Previously, we have demonstrated that polyamines added to the inside (but not the outside) of erythrocyte ghosts prior to resealing inhibited nonspecific, calcium-dependent transbilayer flip-flop of phospholipids (15). These observations prompted us to ask whether alteration of polyamine levels in intact cells during the process of apoptosis would alter the appearance of PS in the outer leaflet of the plasma membrane (8, 14). More specifically, we asked whether polyamine manipulation would affect either calcium-mediated, nonspecific phospholipid flip-flop or loss of aminophospholipid translocase activity, both of which accompany PS appearance in apoptosis of HL-60s (8). Treatment of HL-60s in culture with the ornithine decarboxylase inhibitor, DFMO, resulted in progressive depletion of intracellular spermidine and putrescine over the course of several days. Spermine levels were initially preserved in the presence of DFMO, a pattern typical of other cell types, and known to be secondary to shunting of the other polyamines, putrescine and spermidine, through the polyamine biosynthetic pathway for the synthesis of spermine (34). Beyond the 5th day of incubation with DFMO, spermine content did decline but resulted in unacceptable acceleration of cell death. Experiments were therefore conducted on cultures treated with DFMO for 5 days when nonviable cells (propidium iodide-positive) were noted to be approximately 5%. As shown in Fig. 1, in cells cultured for 5 days with DFMO, both putrescine and spermidine were undetectable (<5 pmol/10^6 cells), whereas spermine was unchanged. Given the close correlation of polyamine levels and cell growth (35), DFMO treatment of cultures resulted in expected slowing of cell proliferation but no alteration in phospholipid classes, protein content, or cell volume (see "Experimental Procedures," data not shown). The addition of putrescine for the final 48 h of culture to bypass the metabolic defect in DFMO-treated cultures spurred proliferation and completely restored spermidine content, although intracellular putrescine remained deficient (Fig. 1). Whereas putrescine is commonly taken up by cells, neither spermidine nor spermine added to DFMO-treated cultures restored intracellular polyamines (34), and both were associated with substantial toxicity by 24 h.

As described previously, UV irradiation (5 min) was used for the rapid induction (2 h) of apoptosis identified by the microscopic appearance of nuclear condensation, cell shrinkage, and plasma membrane zeiosis. Past studies using the caspase inhibitor, Asp-Glu-Val-Asp-fluoromethylketone, have shown complete inhibition of both nuclear and plasma membrane

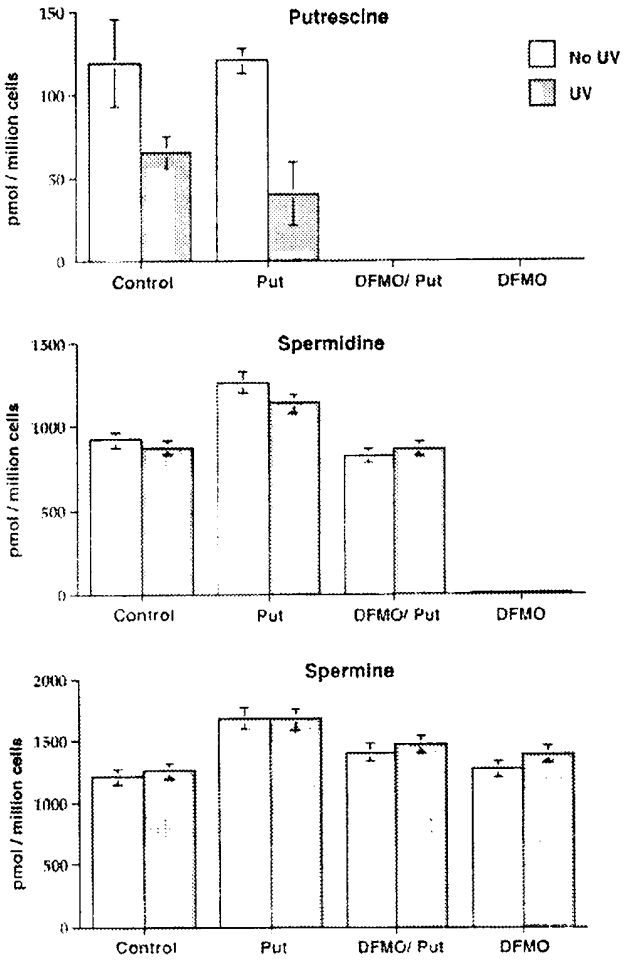


FIG. 1. **Intracellular polyamines (pmol/10^6 cells) in HL-60s incubated with and without DFMO and putrescine in control cells (*open bars*) and in cells following UV irradiation (5 min) and 2 h of culture (*shaded bars*).** Cultures were treated with or without DFMO (1 mM) for 5 days. Putrescine (*Put*) was added (10 μM) as shown for the final 48 h of culture. Data are expressed as means ± S.E. $n = 4$. Note differences in scale for the various polyamines.

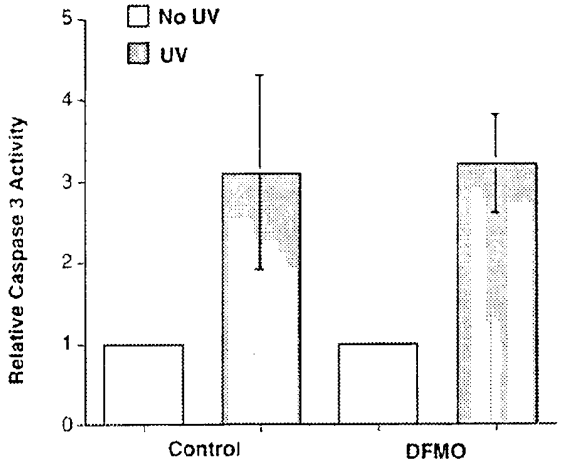


FIG. 2. **Caspase activity in control (*open bars*) and UV-irradiated (*shaded bars*) HL-60s (expressed as fold increase over control).** Cells were incubated with or without DFMO as in Fig. 1. Data are expressed as ± S.E. $n = 3$.

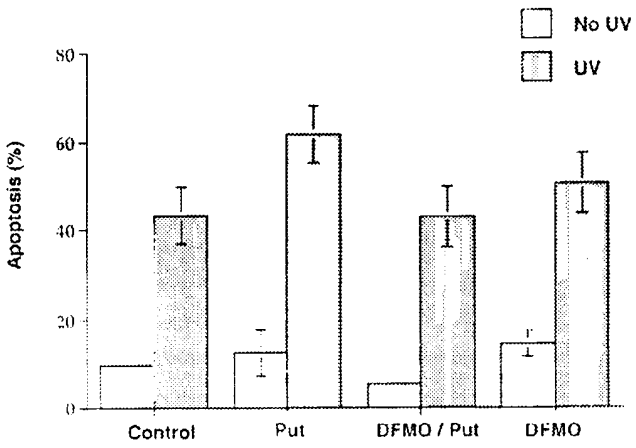


FIG. 3. **Percent of cells undergoing apoptosis as detected by the appearance of hypodiploid DNA in control (*open bars*) and UV-irradiated (*shaded bars*) HL-60s.** Cells were incubated with or without DFMO and putrescine (*Put*) as in Fig. 1. Data are expressed as means ± S.E. $n = 7$.

changes during UV-induced apoptosis of HL-60s, thus affirming the pivotal requirement of caspase activation in the process of apoptosis of these cells. We assessed caspase activity in lysates from DFMO-treated and control cells and found it to be identical at baseline and identically elevated after UV irradiation (Fig. 2). The appearance of hypodiploid DNA following UV irradiation was assessed in the flow cytometer and confirmed by the identification of DNA fragmentation showing characteristic laddering in agarose gels (29). DNA fragmentation in cultured cells was identical regardless of polyamine depletion with DFMO or replenishment with putrescine addition (Fig. 3.) Plasma membrane vesiculation, requiring cytoskeletal assembly and characteristic of the process of zeiosis in apoptosis, was similarly unaffected by culture conditions and polyamine levels (Fig. 4). Thus alteration in polyamine levels had no discernible effect on these fundamental events of apoptosis as follows: caspase activation, DNA fragmentation, or plasma membrane vesiculation.

Of note, the process of apoptosis did not result in the appearance of acetylated polyamines nor was polyamine content grossly altered following induction of apoptosis (Fig. 1, *shaded bars*), as has been reported in some cells (36, 37). A decline in putrescine content was seen in control and putrescine-treated cells, but the significance of this finding is hard to assess as putrescine is a relatively minor polyamine (note scale differences in Fig. 1), and levels were below detectability in DFMO-treated cells with or without putrescine addition.

Loss of aminophospholipid translocase activity, an event thought to modulate the amount of PS detectable on cells undergoing apoptosis (7, 8), was measured by the ability of cells to take up NBD-PS. PS uptake was not altered by either spermidine/putrescine depletion or spermidine repletion (Fig. 5) at either baseline or following UV irradiation. The data, therefore, rule out effects of polyamines on aminophospholipid translocase activity.

In marked contrast, the appearance of PS in the outer leaflet of the plasma membrane was significantly altered by modulating polyamine levels. The appearance of PS, as measured by binding of FITC-labeled annexin V, was completely inhibited when cells were treated with DFMO resulting in depletion of spermidine and putrescine (Fig. 6). Notably, PS appearance was restored in cells undergoing apoptosis when spermidine content was restored by the addition of putrescine during the last 48 h of culture (see Fig. 1). Nonspecific phospholipid flip-flop, previously shown to be a requirement for PS appearance during apoptosis (8), was likewise dependent on polyamine

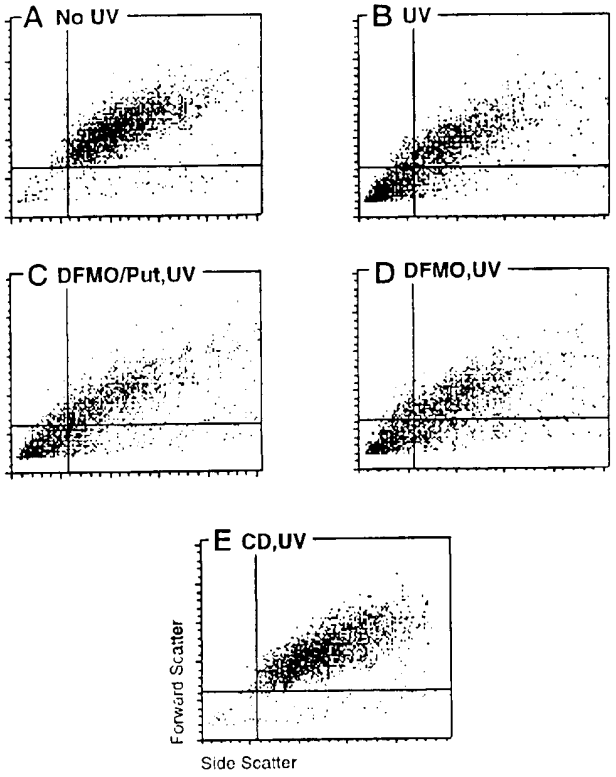


FIG. 4. **Plasma membrane vesiculation as shown by events in the left lower quadrant of forward scatter *versus* side scatter plots for control (*A*), UV-irradiated cells (*B*), UV-irradiated cells following spermidine repletion (DFMO/putrescine) (*C*), UV-irradiated cells following polyamine depletion by DFMO (*D*), and cytochalasin D (*CD*)-treated cells (*E*).** Plots are representative of at least 14 experiments.

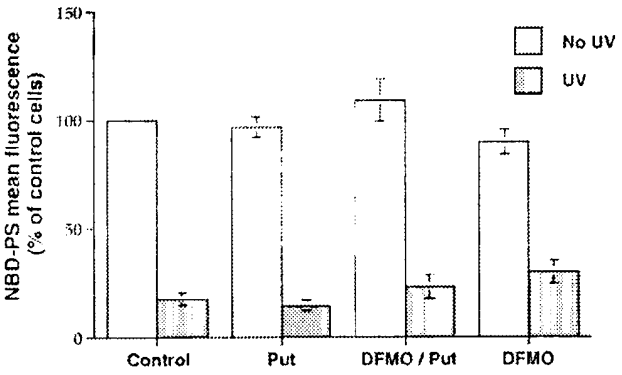
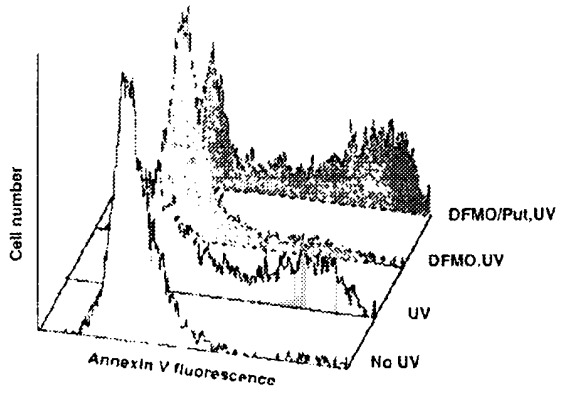


FIG. 5. **Aminophospholipid translocase activity as measured by the uptake of NBD-PS in control (*open bars*) and UV-irradiated (*shaded bars*) HL-60s (expressed relative to mean fluorescence of control cells cultured without DFMO or putrescine).** Cells were incubated with or without DFMO and putrescine (*Put*) as in Fig. 1. Data are expressed as means ± S.E. $n = 7$

status. Nonspecific phospholipid flip-flop as measured by NBD-PC uptake was significantly inhibited in DFMO-treated cells undergoing apoptosis (Fig. 7). Conversely, in cells treated with DFMO followed by putrescine resulting in spermidine repletion, enhanced NBD-PC uptake was restored. To prove that intracellular repletion of spermidine was required for PS appearance and nonspecific phospholipid flip-flop, spermidine (10 μM) was added exogenously for 5 min prior to lipid uptake and found to have no effect. Likewise, putrescine or spermine added externally just prior to lipid uptake had no effect. These



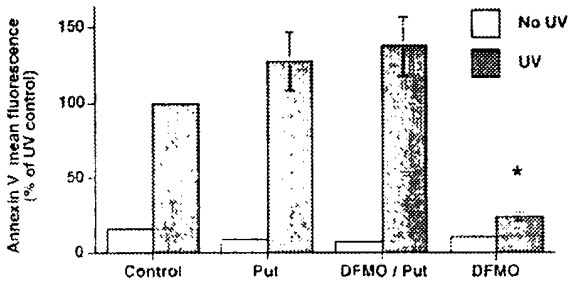


FIG. 6. **Appearance of PS in control and UV-irradiated HL-60s as detected by binding of FITC-labeled annexin V.** *Top*, histograms showing FITC-labeled annexin V binding for control, UV-irradiated cells, UV-irradiated cells following polyamine depletion by DFMO, and UV-irradiated cells following spermidine repletion with DFMO/putrescine (*Put*). *Bottom*, data expressed as mean fluorescence relative to UV-irradiated cells cultured without DFMO or putrescine. Cells were incubated with or without DFMO and putrescine (*Put*) as in Fig. 1. Appearance of PS on UV-irradiated cells cultured with DFMO was no different than control cells without UV irradiation and was significantly less than appearance of PS on UV-irradiated cells cultured without DFMO or with putrescine or DFMO/putrescine added to cultures. Data are expressed as ± S.E. ($n = 9$), $^*p < 0.0001$.

findings demonstrate the requirement for intracellular spermidine repletion for both PS appearance in the plasma membrane outer leaflet and nonspecific phospholipid flip-flop. Taken together, the data affirm our previous findings (8) that during apoptosis loss of aminophospholipid translocase activity alone is insufficient for the appearance of PS (Fig. 5) and that PS appearance correlates with nonspecific phospholipid flip-flop.

As noted above, HL-60s undergo vesiculation and blebbing (zeiosis) during apoptosis regardless of culture conditions (with or without DFMO or putrescine treatment). To address the possibility that vesiculation would alter surface area and consequently the appearance of PS and development of phospholipid flip-flop sites, cytochalasin D, 5 μg/ml, was added to prevent vesiculation (Fig. 4*E*) (8). With or without cytochalasin D added to the cells incubated in the various conditions, the results of lipid uptake were the same, thus controlling for surface area changes and ruling out vesiculation as an event required for either enhanced lipid uptake or the appearance of PS (data not shown).

It has previously been demonstrated that extracellular calcium (ED_{50} 100 μM) is required for the enhanced phospholipid flip-flop that results in PS appearance in the outer membrane leaflet (8, 14) and that HL-60s undergoing apoptosis are capable of transporting calcium across the plasma membrane and of maintaining physiologic calcium concentration (8, 14, 33). Pre-

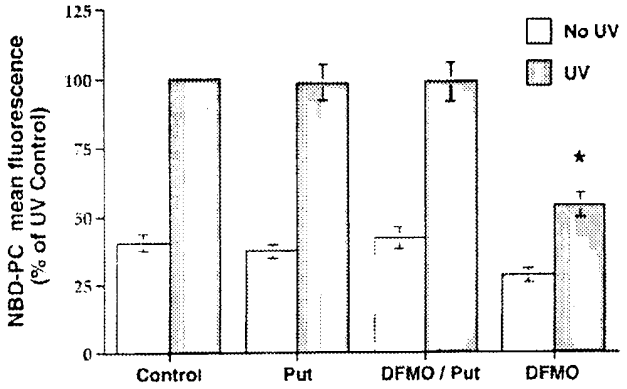


FIG. 7. **Nonspecific transbilayer phospholipid flip-flop as measured by uptake of NBD-PC in control (*open bars*) and UV-irradiated (*shaded bars*) HL-60s (expressed relative to mean fluorescence of UV-irradiated cells cultured without DFMO or putrescine).** Cells were incubated with or without DFMO and putrescine (*Put*) as in Fig. 1. Uptake of NBD-PC in UV-irradiated cells cultured with DFMO was no different than control cells and was significantly less than uptake of NBD-PC in UV-irradiated cells cultured without DFMO or with putrescine added to cultures. $*p < 0.0001$. Data are expressed as ± S.E. $n = 9$.

TABLE I
Intracellular calcium during apoptosis

Intracellular calcium (nM) was determined in control and DFMO-treated HL-60s by the fura-2 method following UV irradiation. After establishing intracellular calcium levels in cells 1 h following UV irradiation, the calcium ionophore, A23187, was added and calcium flux measured. Peak intracellular calcium mobilization is shown. Data are expressed as mean ± S.E., $n = 10$. There were no significant differences in intracellular calcium concentrations between control and DFMO-treated cells.

	Post-UV	A23187
Control	82 ± 7	451 ± 24
DFMO-treated	71 ± 5	544 ± 74

FIG. 8. **Scramblase expression determined by Western blotting (see "Experimental Procedures") in cells incubated in the various conditions.** Data are representative of four experiments.

vious reports of polyamine modulation of membrane permeability and cation flux across membranes (38–40) and, particularly, inhibition of calcium flux from mitochondria implicated in some systems during apoptosis (41–43) prompted us to ask whether DFMO-treated cells with altered polyamine levels would show deficient calcium homeostasis. Intracellular calcium concentrations at baseline, during apoptosis, and mobilization with the calcium ionophore A23187 were identical in both DFMO-treated and control cells (Table I). Thus inhibition of nonspecific phospholipid flip-flop and PS appearance seen in DFMO-treated cells does not appear to be related to altered intracellular calcium homeostasis.

Cellular polyamine levels are highly regulated by multiple metabolic pathways, particularly during cell proliferation and differentiation (34, 35). Alteration of levels of cellular polyamines with DFMO has been known to alter expression and activity of various cellular proteins (44–46). Although the actual protein(s) involved in PS appearance and nonspecific flip-flop have yet to be identified, we assessed the activities or amounts of several proteins implicated in the process of apoptosis and with the enhancement of nonspecific transbilayer movement of phospholipids. First, scramblase mRNA was assessed using quantitative PCR. Scramblase message was present in DFMO-treated cells at approximately 50% that of untreated cells (Fig. 8*A*). However, scramblase mRNA was not increased in cells treated with DFMO followed by exogenous putrescine addition despite restoration of PS appearance and calcium-mediated nonspecific flip-flop. Importantly, expression of scramblase protein, as detected by Western blotting, was only slightly diminished with DFMO treatment and was unchanged with putrescine addition following DFMO treatment (Fig. 8). Similarly, transglutaminase activity was measured in lysates from DFMO-treated and control cells. As has been shown previously (47), little activity was demonstrated in these undifferentiated HL-60s (relative to differentiated HL-60s and other cells), and no differences were demonstrated between DFMO-treated cells and control cells (data not shown). Bcl-2, a survival protein (48), was also assessed by Western blotting, as an increase in Bcl-2 might confer resistance to apoptotic membrane changes in DFMO-treated cells. Again, no differences were demonstrated between DFMO-treated and control cells (data not shown). In a final attempt to show that the effects of DFMO treatment and subsequent spermidine repletion were attributable to the polyamine manipulation, and not a particular target protein, we treated cells with cycloheximide to inhibit protein synthesis during incubation with putrescine. As shown in Fig. 9, cycloheximide treatment neither altered the loss of PS appearance following UV irradiation in DFMO-treated cells nor altered the restoration of PS appearance following spermidine repletion. In these experiments, we observed that treatment with cycloheximide did not have any effect on spermine or spermidine levels following DFMO treatment, with or without putrescine addition; they remained the same as those shown in Fig. 1 (without cycloheximide). However, in the presence of cycloheximide, putrescine levels as well as spermidine levels were restored in cells incubated with putrescine. These experiments demonstrate that although individual polyamines cannot be manipulated as in simplified membrane models, alterations in polyamine levels, particularly spermidine, in intact cells can govern PS appearance and nonspecific flip-flop during cellular apoptosis (see "Discussion").

DISCUSSION

In earlier work from this laboratory, we have shown that enhanced phospholipid flip-flop and PS appearance seen during apoptosis are entirely dependent on the presence of extracellular calcium (8). Conversely, DNA fragmentation, plasma membrane vesiculation, and loss of the aminophospholipid translocase activity do not require extracellular calcium. Significantly, this dichotomy of events during apoptosis is also seen with regard to DFMO treatment. The events dependent on extracellular calcium, nonspecific phospholipid flip-flop, and PS appearance are also inhibited by DFMO treatment and restored with spermidine repletion (Figs. 1, 6, 7, and 9). Conversely, the events independent of extracellular calcium, DNA fragmentation, plasma membrane vesiculation, and loss of the aminophospholipid translocase activity, proceed during apoptosis independently of whether polyamines are altered with DFMO treatment or not (Figs. 3–5). Calcium mobilization does not differ in DFMO-treated *versus* control cells (Table I), and thus, altered calcium mobilization does not explain the inhibition of phospholipid flip-flop and PS appearance seen with DFMO treatment. On the other hand, polyamines are known to

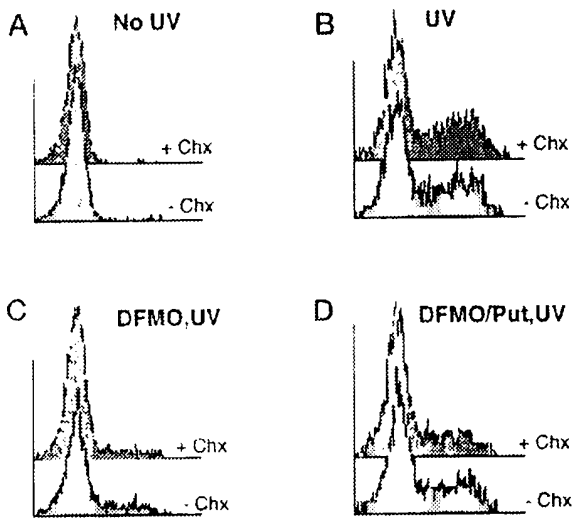


FIG. 9 **Histograms showing the appearance of PS as detected by FITC-labeled annexin V for control (*A*), UV-irradiated cells (*B*), UV-irradiated cells after polyamine depletion by DFMO (*C*), and UV-irradiated cells after spermidine repletion (DFMO/putrescine) (*D*).** ***Rear histograms*** show cells for each condition cultured identically but with the inclusion of cycloheximide during the final 20 h of culture. Plots are representative of seven experiments.

antagonize the effects of divalent cations in many models (49). The placement of DFMO treatment acting downstream of the requirement for calcium (8) would be consistent with the data. In support of this, in a simplified erythrocyte ghost model polyamines in a concentration-dependent fashion inhibit calcium-induced phospholipid flip-flop, inhibition that is overcome by increasing calcium concentration suggesting that polyamines screen calcium from "active flip sites" (15, 28). However, inhibition of phospholipid flip-flop in the erythrocyte ghost model demonstrates rank ordering of inhibition by spermine > spermidine ≫ putrescine (15). Here we demonstrate that depletion of spermidine and putrescine results in a significant decrease in phospholipid flip-flop as measured by both appearance of endogenous PS and uptake of exogenously added NBD-PC following induction of apoptosis (Figs. 6 and 7). How then can we reconcile the data from simplified membrane models, where levels of each polyamine can be manipulated independently, with the current data in intact cells undergoing apoptosis where spermidine and putrescine are depleted together (without spermine being affected) and spermidine alone repleted? Several possibilities are suggested. First, spermidine and putrescine have been shown to antagonize spermine (49). As such, DFMO treatment, resulting in putrescine and spermidine depletion, may leave a relative enrichment of spermine that could result in enhanced screening of calcium from either anionic phospholipids such as phosphatidylinositol bisphosphate (28) and/or proteins that act as flippases or scramblase(s) (17–19, 50). Second, intracellular pools of polyamines (particularly cytoplasmic or submembranous) may not be reflected in bulk intracellular measures and may be critical to the modulating enhanced phospholipid flip-flop during apoptosis. Third, addition of putrescine to cultures resulting in spermidine repletion restored enhanced phospholipid flip-flop and PS appearance. Thus spermidine may be required in a permissive fashion for the function of a flippase. Further definition of the relative contributions of the various polyamines awaits both more specific means to alter polyamine species and definition of potential targets modulated by polyamines.

Aside from screening of calcium, there are other functions of polyamines that may be relevant to explain the data. At physiologic pH, the amino groups of the polyamines are protonated and bind negatively charged macromolecules including nucleic acids, membrane and cytoskeletal proteins, and anionic phospholipids. Consequences of polyelectrolytic binding, however, are not simply a matter of charge number (15, 49) but reflect charge density dictated by the distances between the amines located along the flexible carbon chain. Work by Chung *et al.* (51) and Meers *et al.* (52) determined that spermine is oriented parallel to the membrane bilayer forming a multivalent complex with anionic phospholipids. Polyamines with the rank order of spermine > spermidine > putrescine have been shown to "freeze" membranes by their charge interactions with both anionic phospholipids (28, 53) and cytoskeletal proteins functioning as bridging elements (54). Implications of this binding include steric hindrance of the bound membrane constituents, rigidification of the bilayer polar region, and possible phase separation of lipids in the membrane inner leaflet. As such, we would hypothesize that spermine, without antagonism by the less charged polyamine species, spermidine or putrescine, may inhibit lateral mobility of membrane proteins that act as phospholipid flip sites (17, 19, 55, 56) as well as phospholipids that would otherwise be flipped after gaining access to such proteins. Alternatively, spermine could enhance membrane-cytoskeletal interaction by minimizing the electrostatic repulsion of the membrane and spectrin (fodrin) (57), a protein whose cleavage is associated with PS appearance during apoptosis (24, 58). Recently, a 37-kDa protein, the phospholipid scramblase, has been implicated in enhanced phospholipid flip-flop in platelets and the erythrocyte model (18). This scramblase has been recently cloned, purified, and shown to mediate calcium-mediated phospholipid flip-flop (albeit at relatively low levels) when reconstituted in proteoliposomes (19, 50). Notably, this protein when isolated from erythrocytes and reconstituted in vesicles does not appear to be regulated by polyamines (59). Of interest, HL-60s, unlike several other cell lines and peripheral blood leukocytes, appear to have relatively little expression of the scramblase protein (20). HL-60s, nonetheless, do express PS similarly to other cells undergoing apoptosis (8) implying either that existing scramblase activity is adequate or that there exist other candidates for moving PS into the outer leaflet. We note that DFMO treatment did reduce scramblase mRNA expression by approximately 50%, but putrescine treatment, while restoring PS appearance and phospholipid flip-flop, did not increase scramblase mRNA. Scramblase protein expression was affected minimally by DFMO treatment, with or without putrescine addition (Fig. 8). Alternative candidates for flippase are members of the multidrug resistance P-glycoprotein family (17). However, unlike phospholipid flip-flop mediated by the multidrug resistance proteins, we were unable to inhibit enhanced flip-flop with verapamil suggesting the multidrug resistance proteins are not candidates in this system (8, 17). Additionally, other as yet unidentified proteins have been noted to exhibit the ability to effect phospholipid transbilayer movement (55, 56). Thus which candidate flippase is active in HL-60s undergoing apoptosis and whether it, or modulators of its activity (60, 61), interacts with polyamines is the goal of future studies. We note that several proteins, protein kinase CK2 (46, 62) and the inward rectifier potassium channel (38, 63, 64), have binding sites for spermine and are specifically regulated by it, thus setting precedence for polyamine regulation of protein function.

Finally, from these data in HL-60s and in previous data from neutrophils and Jurkat cells (8), it appears that whereas loss of the aminophospholipid translocase may enhance the appearance of PS, calcium-mediated phospholipid flip-flop is required for PS appearance on the surface of apoptotic cells. Although

we were unable to modulate specifically the individual polyamines, or to deplete spermine, as can be done using model systems, we were able for the first time to show that modulation of polyamine levels in living cells inhibited PS appearance and calcium-mediated nonspecific flip-flop. Recognition of the general finding that PS appears in the plasma membrane outer leaflet of cells undergoing apoptosis (3, 8, 9) and that PS serves as a signal to phagocytes for distinctive, "noninflammatory" engulfment of apoptotic cells (65–68) underscores the importance of defining the underlying mechanism(s) for PS appearance.

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